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Differential Consequences of Two Distinct AhR Ligands on Innate and Adaptive Immune Responses to Influenza A Virus

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Immune modulation by the aryl hydrocarbon receptor (AhR) has been primarily studied using 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD). Recent reports suggest another AhR ligand, 6-formylindolo[3,2-b]carbazole (FICZ), exhibits distinct immunomodulatory properties, but side-by-side comparisons of these 2 structurally distinct, high-affinity ligands are limited. In this study, the effects of *in vivo* AhR activation with TCDD and FICZ were directly compared in a mouse model of influenza virus infection using 3 key measures of the host response to infection: pulmonary neutrophilia, inducible nitric oxide synthase (iNOS) levels, and the virus-specific $CD8^+$ T-cell response. By this approach, the consequences of AhR activation on innate and adaptive immune responses to the same antigenic challenge were compared. A single dose of TCDD elicited AhR activation that is sustained for the duration of the host's response to infection and modulated all 3 responses to infection. In contrast, a single dose of FICZ induced transient AhR activation and had no effect on the immune response to infection. Micro-osmotic pumps and *Cyp1a1*-deficient mice were utilized to augment FICZ-mediated AhR activation *in vivo*, in order to assess the effect of transient versus prolonged AhR activation. Prolonged AhR activation with FICZ did not affect neutrophil recruitment or pulmonary iNOS levels. However, FICZ-mediated AhR activation diminished the $CD8^+$ T-cell response in *Cyp1a1*-deficient mice in a similar manner to TCDD. These results demonstrate that immunomodulatory differences in the action of these 2 ligands are likely due to not only the duration of AhR activation but also the cell types in which the receptor is activated.

Key Words: **AhR; TCDD; FICZ; influenza virus.**

It is widely accepted that the aryl hydrocarbon receptor (AhR) influences the development and function of the immune system, although the molecular mechanisms through which AhR does this remain to be fully elucidated. The majority of these studies have used persistent environmental contaminants, including polycyclic aromatic hydrocarbons, halogenated aromatic hydrocarbons, and some polychlorinated biphenyls to activate the AhR (Burchiel and Luster, 2001; Lawrence and Kerkvliet, 2006; Nguyen and Bradfield, 2008). Other studies have used naturally derived chemicals, such as the photodegradation product of tryptophan, 6-formylindolo[3,2-b] carbazole (FICZ), or novel pharmaceutical candidates, such as VAF347, to activate AhR and modulate immune responses (Lawrence *et al.*, 2008; Quintana *et al.*, 2008; Veldhoen *et al.*, 2008). To date, the best characterized and most commonly used AhR ligand is the pollutant 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD). Indeed, the majority of published studies investigating immune modulation by AhR have used TCDD (Lawrence and Kerkvliet, 2006). Reasons for this include TCDD's high affinity for the AhR, its demonstrated specificity for the receptor, and its long half-life *in vivo* (Bohonowych and Denison, 2007; Gasiewicz *et al.*, 1983; Mimura *et al.*, 1997; Safe, 1990). However, using TCDD is often criticized because the same properties that make it an excellent tool for activating AhR also contribute to its known toxicity (Nguyen and Bradfield, 2008). Furthermore, given that TCDD is a synthetic chemical, tremendous interest lies in characterizing the immunomodulatory potential of naturally derived chemicals and putative endogenous ligands for the AhR in an effort to better understand the role this receptor plays in immune function.

Based principally on information derived from studies using TCDD, we know the AhR rests as an inactive complex in the cytosol, which upon ligand binding dissociates from cochaperone proteins, translocates into the nucleus, and binds to its heterodimerization partner: hypoxia inducible factor-1β (HIF-1β, also called AhR nuclear translocator). The ligand:AhR:HIF-1β complex binds to aryl hydrocarbon response elements in the upstream regulatory region of AhR target genes (Abel and Haarmann-Stemmann, 2010). Although the AhR has been shown to *directly* regulate the expression of about 25 different genes (Gasiewicz *et al.*, 2008; Tijet *et al.*, 2006), the phase I drug-metabolizing enzymes, cytochrome p450 (CYP) 1A1, 1A2, and 1B1, are often used as biomarkers of AhR

activation (Fujii-Kuriyama and Mimura, 2005; Nebert *et al.*, 2004; Schrenk, 1998; Vanden Heuvel *et al.*, 1993).

Consistent immunomodulatory effects of AhR activation by TCDD have been reported in assorted rodent models using a variety of different antigens and measuring altered functions of several immune cell types (reviewed in Stevens, 2009). Epidemiological reports reveal the adverse effects of man-made dioxins and dioxin-like chemicals on human health (Dallaire *et al.*, 2006; Stolevik *et al.*, 2011; Van Den Heuvel *et al.*, 2002). However, several studies have shown that the nonanthropogenic AhR ligand FICZ can also alter immune function. For example, FICZ treatment skews $CD4^+$ T-cell differentiation and enhances the severity of experimental autoimmune encephalomyelitis (EAE), a mouse model that mimics aspects of multiple sclerosis (Quintana *et al.*, 2008; Veldhoen *et al.*, 2008). Intriguingly, in the same disease model, TCDD-mediated AhR activation promoted regulatory T-cell (T_{reg}) differentiation and reduced the development of EAE (Quintana *et al.*, 2008). These findings raise the possibility that depending on the type of ligand used to activate the AhR, dramatic differences in immune outcomes can occur. However, it has not been established whether differential immune modulation is unique to particular disease models, such as EAE, or to $CD4^+$ T-cell responses, or whether this dichotomy would be observed in other disease model systems or in the context of the responses of other types of cells.

In the work reported here, we directly compared the consequences of AhR activation by the pollutant TCDD and the potential endogenous AhR ligand FICZ on 3 key immune responses to infection with human influenza A virus: pulmonary neutrophilia, inducible nitric oxide synthase (iNOS) levels in the lung, and virus-specific $CD8^+$ cytotoxic T lymphocytes (CTL). Modulation of these responses to infection is mediated specifically through AhR activation (Lawrence *et al.*, 2006; Neff-LaFord *et al.*, 2007; Teske *et al.*, 2008). Moreover, these particular responses play critically important roles in host resistance to primary influenza A virus infection (Akaike *et al.*, 1996; Flynn *et al.*, 1998; Tate *et al.*, 2011) and represent 3 distinct pathways through which AhR modulates host responses to infection (Lawrence *et al.*, 2006; Wheeler *et al.*, 2013). Further, using micro-osmotic pumps and *Cyp1a1*-deficient mice, we investigated the consequences of the duration and nature, or "quality," of AhR activation on these responses to influenza virus infection in an effort to better characterize specifically how these 2 structurally distinct AhR ligands impact viral immunity.

MATERIALS AND METHODS

Animals and treatment. C57BL/6 mice (female, 6–10 weeks of age) were purchased from either the Jackson Laboratories or the National Cancer Institute. Breeding stock for *Cyp1a1*-deficient (*Cyp1a1*$^{-/-}$) mice were kindly provided by Dr Daniel Nebert (University of Cincinnati) and were backcrossed onto the C57BL/6 background (Dalton *et al.*, 2000). All mice were housed in pathogen-free microisolator cages and maintained on a 12-h light/dark cycle, and provided food and water *ad libitum.* Unless indicated otherwise, mice were gavaged with either 10 μg TCDD/kg body weight (≥ 98% pure, Cambridge Isotope Laboratories) dissolved in anisole and diluted in peanut oil or 10 μg FICZ/kg body weight (Enzo Life Sciences, Farmingdale, New York) dissolved in dimethysulfoxide (DMSO) and diluted in peanut oil. Anisole diluted in peanut oil was used as the vehicle control. To overcome transient AhR activation by FICZ, for some experiments, micro-osmotic pumps (Alzet, Cupertino, California, Model 1007D) were implanted 1 day prior to infection. The pumps continuously release FICZ at a rate of 10 μg/kg body weight/h until the day of sacrifice; thus, mice are exposed to FICZ continuously. For the micropumps, FICZ was dissolved in DMSO and diluted in 45% hydroxypropyl-β-cyclodextrin (HPβCD; wt/vol, water) or vehicle control (diluted HPβCD). One day after gavage or pump implantation, mice were anesthetized with Avertin (2,2,2-tribromoethanol) and infected intranasally with 120 hemagglutinating units influenza virus, strain A/HKx31 (x31, H3N2). This dose of virus is typically sublethal in vehicle-treated, immunocompetent mice (Vorderstrasse *et al.*, 2003). The University of Rochester Institutional Animal Care and Use and Institutional Biosafety Committees preapproved all procedures involving laboratory animals and infectious agents.

Immune cell isolation. For lung-derived immune cells, the left and right lung lobes were separated at the bronchi. For all experiments, the same side was snap frozen in liquid nitrogen and used for immunoblotting or reverse transcription-PCR (RT-PCR). The other side was used to obtain lung-derived immune cells and lung lavage fluid. Collagenase digestion of lung tissue was performed as previously described (Teske *et al.*, 2005). Immune cell isolation from the lung-draining mediastinal lymph nodes was performed 9 days postinfection as previously described (Lawrence *et al.*, 2006). Erythrocytes were lysed using ammonium chloride and total organ-derived immune cells were enumerated using a Coulter counter (Beckman Coulter Corp., Miami, Florida), TC10 automated cell counter (Bio-Rad, Hercules, California), or hemacytometer.

Immunophenotypic analyses. Nonspecific staining was blocked using anti-mouse anti-CD16/32 (eBioscience, San Diego, California) and rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania). Influenza A virus (HKx31)–specific $CD8^+$ T cells were identified using an major histocompatibility complex class I tetramer with a viral peptide, $D^b/NP_{366–374}$, as previously described (Lawrence *et al.*, 2006). The $NP_{366–374}$ peptide is one of the major immunodominant epitopes of HKx31 virus detected by $CD8^+$ T cells from C57BL/6 mice (Townsend *et al.*, 1986). Cells were incubated with the fluorochrome-conjugated tetrameric reagent prior to labeling with fluorochrome-conjugated antibodies directed against CD8, CD44, and CD62L (eBioscience or BD Biosciences, San Jose, California). To identify interferon (IFN)γ-producing cells, isolated cells were restimulated *ex vivo* for 5 h with 12.5 U/ml recombinant mouse interleukin-2, 1 μM influenza virus nucleoprotein peptide ($NP_{366–374}$, ASNENMETM), and 5 mg/ml brefeldin A (Neff-LaFord *et al.*, 2007). Restimulated cells were stained with fluorochrome-conjugated monoclonal antibodies, fixed, permeabilized, and incubated with a fluorochrome-conjugated anti-IFNγ antibody (eBioscience). Lung-derived immune cells were incubated with fluorochrome-conjugated monoclonal antibodies directed against CD45, CD11b, Gr-1, and CD8 (eBioscience or BD Biosciences). Data were collected using an LSR II 12-color flow cytometer (BD Biosciences; at least 100 000 cells were collected for neutrophil phenotypic analyses, and 300 000–500 000 cells were collected for T-cell analyses) and analyzed using FlowJo software (Treestar, Ashland, Oregon). Neutrophils (and other leukocytes) were also enumerated by differential cell counting of hematoxylin and eosin–stained lung-derived immune cells, as previously described (Teske *et al.*, 2008).

Immunoblotting. Frozen lung lobes were homogenized in cold homogenization buffer with protease inhibitors using a Tissue Tearor (Biospec Products, Bartlesville, Oklahoma), and the protein concentration of clarified homogenates was determined by bicinchoninic acid protein assay (Pierce, Rockford, Illinois). Samples were boiled in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) buffer for 5 min and 50 μg of protein was subjected

to SDS-PAGE. After transfer to PVDF membranes, blots were blocked with 5% nonfat dry milk and incubated with anti-iNOS (Cayman, Ann Arbor, Michigan), anti-CYP1A1 (Santa Cruz Biotechnology, Santa Cruz, California), or anti-β-actin (Sigma-Aldrich, St Louis, Missouri) antibodies. After incubation with appropriate horseradish peroxidase–conjugated secondary antibodies, immunoblots were developed with enhanced chemiluminescent reagent (Western Lightning Plus ECL, Pierce) and exposed to x-ray film.

RNA isolation and quantification. Lungs or livers were homogenized in Trizol and total RNA was isolated (Ambion RNA isolation kit, Life Technologies, Grand Island, New York) using the manufacturer's instructions. RNA (1 µg) was reverse transcribed using Oligo (dT) Primers (RETROscript, Ambion) and M-MLV enzyme (Ambion). Quantitative RT-PCR was performed using the MyIQ2 with SYBR green (Bio-Rad) using the following primers: *Cyp1a1*: forward 5′ CTG CCA ATC ACT GTG TCT A 3′; reverse 5′ TTT GGA GCT GGG TTC GAC AC 3′; *Gapdh*: forward 5′ GAA CAT CAT CCC TGC ATT C 3′; reverse 5′ CCA GTG AGC TTC CCG TTC A 3′; *L13*: forward 5′ CTA CAG TGA GAT ACC ACA CCA AG 3′; reverse 5′ TGG ACT TGT TTC GCC TCC TC 3′. The fold change in *Cyp1a1* was calculated relative to the same-day vehicle control and normalized to the reference gene *L13* or *Gapdh* using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

AhR activity. H1L1.1c2 mouse hepatoma cells (gift of Michael Denison, University of California at Davis) that are stably transfected with an AhR-driven reporter construct were used to directly compare the activity of FICZ and TCDD. H1L1.1c2 cells were propagated and the assay was set up as previously described (Ziccardi *et al.*, 2000). Briefly, confluent monolayers of H1L1.1c2 cells in 96-well plates were treated with the indicated concentrations of TCDD or FICZ. Control wells of H1L1.1c2 cells were cultured in media containing the vehicle (0.1% DMSO), and AhR-driven luciferase was measured 18 h later using an automated microplate luminometer (Spectra Max M5, Molecular Devices, Sunnyvale, California).

Statistical analysis. All statistical analyses were conducted using StatView statistical software (SAS, Cary, North Carolina). Differences between the treatment groups were analyzed using 1-way ANOVA, followed by Bonferroni-Dunn *post hoc* test. Differences between 2 treatment groups on a single day postinfection were analyzed using a Student's *t* test. A value of $p \leq .05$ was considered significant.

RESULTS

AhR Activation by FICZ Does Not Elicit the Same Changes in the Immune Response to Influenza Virus Infection That Are Observed With TCDD

To directly compare the consequences of AhR activation by TCDD and FICZ, we administered the same dose of each ligand by oral gavage and examined pulmonary neutrophilia, iNOS levels in the lung, and $CD8^+$ T-cell responses to influenza virus infection. The 7th day after infection is the point in time in which AhR-mediated increases in neutrophilia and iNOS are readily observed in the lung (Neff-LaFord *et al.*, 2007; Wheeler *et al.*, 2013). In contrast to TCDD, FICZ did not enhance the frequency of neutrophils or levels of iNOS in the infected lung (Fig. 1). Additionally, a one-time oral dose of FICZ administered 1 day prior to infection did not increase CYP1A1 protein levels in the lung 7 days after infection, which is in contrast to sustained CYP1A1 induction observed after a single dose of TCDD (Fig. 1B). Likewise, in contrast to the profoundly suppressive effect of TCDD, a single dose of FICZ did not alter influenza virus–specific $CD8^+$ T-cell clonal expansion (Figs. 2A and 2B) and differentiation (Figs. 2C and 2D). We repeated these comparisons using 10-fold higher oral dose of FICZ (100 µg/kg) and again observed no effect on immune responses to influenza virus, as opposed to the immune modulation caused by treating infected mice with 10 µg/kg TCDD (data not shown).

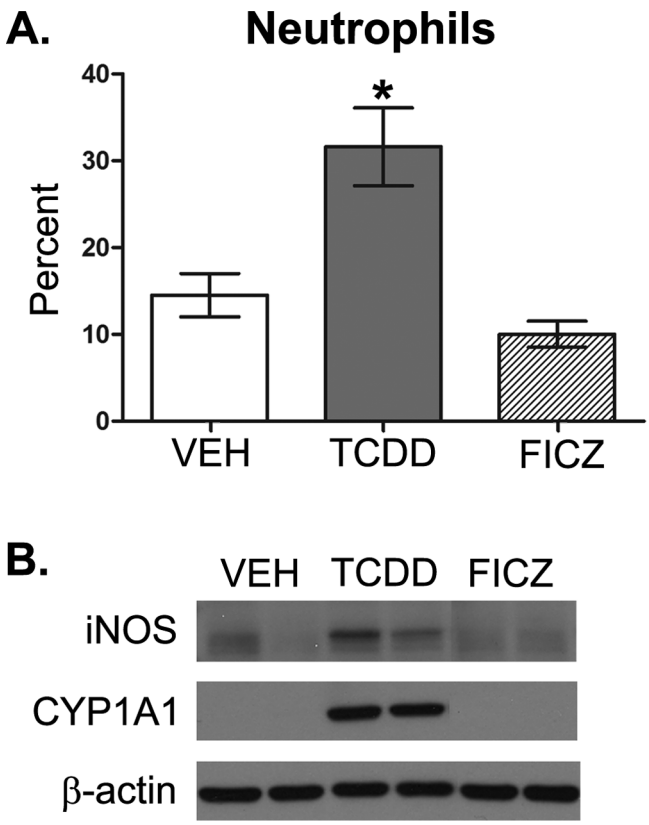

FIG. 1. AhR activation by TCDD and FICZ differentially affects immune responses to influenza virus infection in the lung. Female C57BL/6 mice (8 weeks of age) were gavaged with 10 µg/kg body weight TCDD (T), 10 µg/kg FICZ, or peanut oil anisole control (VEH). One day later, mice were infected intranasally with 120 hemagglutinating units of influenza virus (strain HKx31, H3N2). Mice were sacrificed 7 days postinfection. A, The frequency of neutrophils was determined by differential cell counts of cytospins of airway cells obtained by bronchoalveolar lavage. Bars depict the average percentage of neutrophils (± SEM) for each treatment group (n = 6–7 mice per treatment group). An * indicates a significant difference compared with vehicle control ($p \leq .05$). B, Lung homogenates were subjected to SDS-PAGE and probed for iNOS and CYP1A1. β-Actin levels were assessed as a control. Blots show 2 representative samples from each treatment group (n = 6–7 mice per treatment group). Abbreviations: AhR, aryl hydrocarbon receptor; FICZ, 6-formylindolo[3,2-b]carbazole; iNOS, inducible nitric oxide synthase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin; VEH, vehicle.

AhR Activation Is More Transient With a Single Dose of FICZ Compared With a Single Dose TCDD

In C57BL/6 mice, the *in vivo* half-life of TCDD is about 11 days (Gasiewicz *et al.*, 1983); however, the *in vivo* half-life of

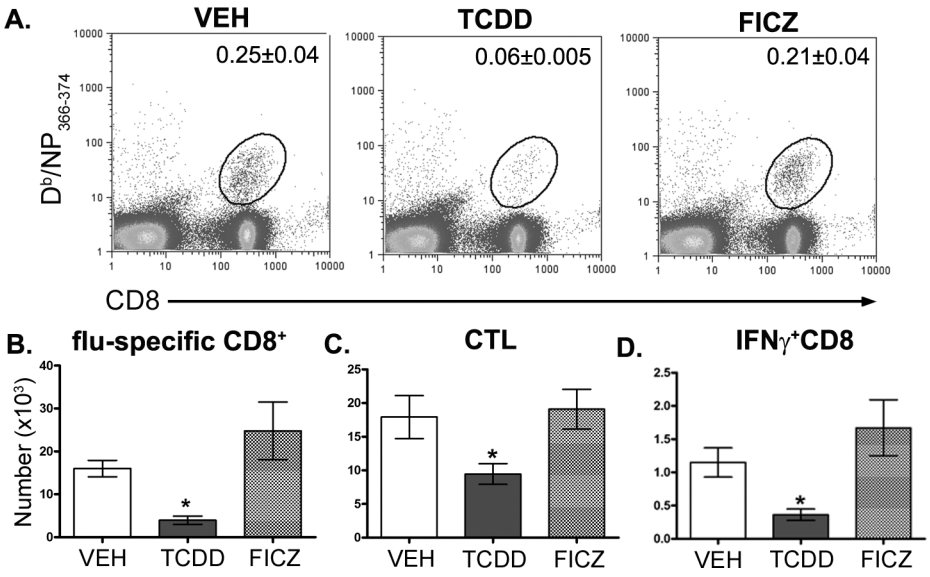

FIG. 2. AhR activation by FICZ does not impair the CD8⁺ T-cell responses to influenza virus infection. Mice were treated and infected as described in Figure 1. On day 9 postinfection, MLN cells were collected, stained with MHC class I-restricted tetramers and antibodies to cell surface proteins, and analyzed by flow cytometry. A, Representative dot plots depict the frequency of $D^b/NP_{366–374}$-specific (flu-specific) CD8⁺ T cells. The number on each plot indicates the average percentage of total MLN cells for each treatment group (± SEM, $n = 5$ mice per treatment group). Bars represent the average number of (B) $D^b/NP_{366–374}$-specific CD8⁺ cells (flu-specific), (C) $CD44^{hi}CD62L^{lo}CD8^+$ cells (CTL), and (D) IFNγ⁺CD8⁺ cells in each treatment group (± SEM; $n = 5$ mice per treatment group). An * indicates a significant difference in cell number compared with vehicle control ($p \leq .05$). Abbreviations: AhR, aryl hydrocarbon receptor; CTL, cytotoxic T lymphocyte; FICZ, 6-formylindolo[3,2-b]carbazole; IFN, interferon; MHC, major histocompatibility complex; MLN, mediastinal lymph node; NP, nucleoprotein; VEH, vehicle.

FICZ in mice remains to be formally determined. Like TCDD, FICZ is a potent inducer of *Cyp1a1*, *1a2*, and *1b1* (Wei *et al.*, 1998; Wincent *et al.*, 2009). However, unlike TCDD, FICZ is readily metabolized, largely by CYP1A1 (Mukai and Tischkau, 2007), which suggests that the *in vivo* half-life of FICZ is likely shorter than TCDD's. Using the induction of CYP1A1 as a measure of AhR activation, we compared a single oral dose (10 µg/kg) of either TCDD or FICZ administered 1 day prior to infection. In mice given TCDD, increased CYP1A1 protein levels were observable in the lung 7 days after infection (Fig. 3A). However, there was no difference in CYP1A1 levels in lungs of mice that received a single oral dose of FICZ (Fig. 3A, lanes labeled F1). Moreover, administering 10 µg/kg FICZ twice daily starting 1 day prior to, and continuing until 7 days after infection, did not result in detectable CYP1A1 levels in the lung (Fig. 3A, lanes labeled F2). In separate studies, we directly compared the ability of FICZ and TCDD to activate the AhR. Using a mouse hepatoma cell line that has been stably transfected with a luciferase gene under the control of the *Cyp1a1* promoter, FICZ and TCDD elicit similar levels of AhR activation in an *in vitro* system (Fig. 3B). Collectively, this information suggests that FICZ results in transient AhR activation *in vivo*, whereas TCDD treatment with the same oral dose results in sustained receptor activation.

To test this idea further, we examined *Cyp1a1* levels several hours after AhR ligand exposure (Fig. 3C). Compared with mice given the vehicle control, increased *Cyp1a1* expression was discernible 1 and 6 h after gavage with 10 µg/kg FICZ. However, by 9 h, *Cyp1a1* levels were not different from vehicle-treated mice. In contrast, *Cyp1a1* expression is still clearly elevated 9 h later in mice administered the same oral dose of TCDD (Fig. 3C). When treated with 10-fold more FICZ (100 µg/kg), *Cyp1a1* levels were qualitatively higher and persisted longer than when mice were treated with the lower dose; however, 18 h after treatment, *Cyp1a1* levels were not different than vehicle-treated mice (Fig. 3D). Together, these data suggest that a single oral dose of TCDD results in AhR activation being sustained for the duration of the immune response to infection (ie, up to at least 9 days), whereas a single dose of FICZ transiently activates AhR, with the effect waning in less than 18 h. This implies that sustained rather than transient activation of the AhR may be necessary to observe altered immune responses to influenza virus over the course of the infection.

Prolonged FICZ-Mediated AhR Activation During Infection Does Not Alter the Response to Infection That Are Modulated When AhR Is Activated Using TCDD

To test whether the duration of AhR activation influences immune responses to influenza virus, we sought to prolong FICZ-mediated AhR activation *in vivo*. To ensure the delivery of a consistent dose of FICZ during infection, micro-osmotic pumps were used to administer 10 µg FICZ/kg body weight/h throughout the course of infection. To monitor whether AhR activation was prolonged by administering FICZ in this manner, *Cyp1a1* expression was measured during the course of

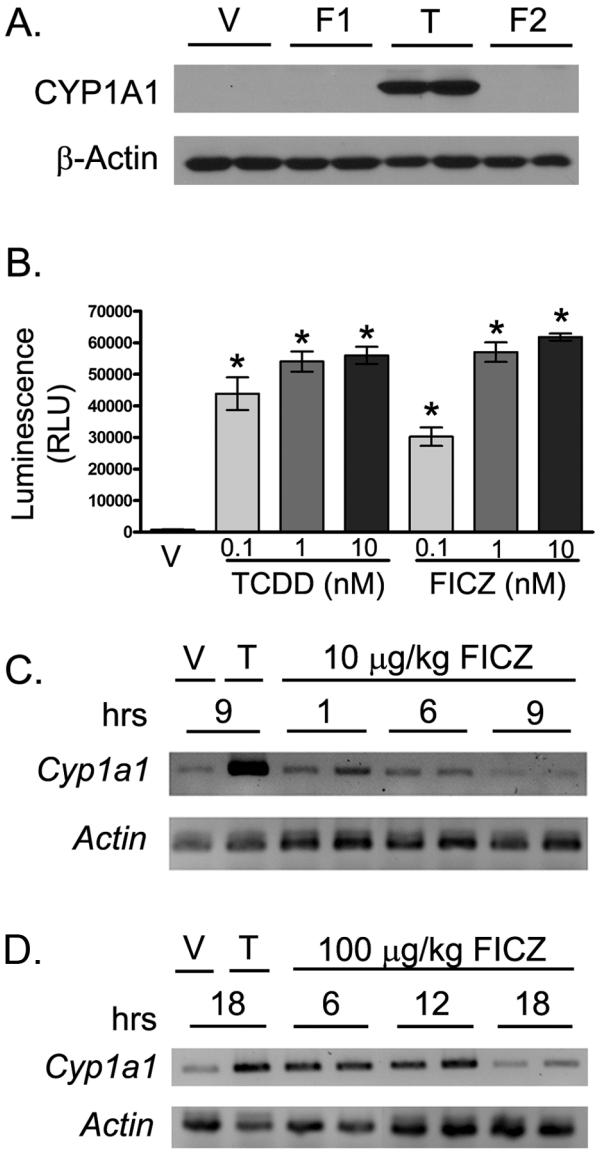

FIG. 3. FICZ is a more transient activator of the AhR than TCDD. A, C57BL/6 mice were gavaged with 10 µg/kg TCDD, 10 µg/kg FICZ, or peanut oil vehicle 1 day prior to infection. Mice receiving FICZ were dosed either once a day (F1) or every 12 h (F2) until day 7 postinfection. Lung homogenates were subjected to SDS-PAGE and probed for CYP1A1. β-Actin levels were assessed as a control. Blots show 2 representative samples from each treatment group (n = 6–7 mice per treatment group). B, Triplicate wells of H1L1.1c2 cells (2×10^5 cells/well) were cultured overnight in media containing 0.1% DMSO (V) or the indicated concentration of TCDD or FICZ. The graphs depict the mean (± SEM) luciferase activity in each treatment group. C and D, C57BL/6 mice (2–3 per group) were gavaged with vehicle control, 10 µg/kg TCDD (C), 10 µg/kg FICZ (C), or 100 µg/kg FICZ (D), and *Cyp1a1* gene expression levels were determined examined at the indicated points in time (hours) relative to treatment using RT-PCR, as described in the Materials and Methods section. PCR products were run on an agarose gel and visualized by ethidium bromide staining. Representative data are shown, where T = TCDD and V = vehicle. An * indicates a significant difference in compared with vehicle control group ($p \leq .05$). Abbreviations: AhR, aryl hydrocarbon receptor; DMSO, dimethysulfoxide; FICZ, 6-formylindolo[3,2-b]carbazole; RT-PCR, reverse transcription-PCR; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin.

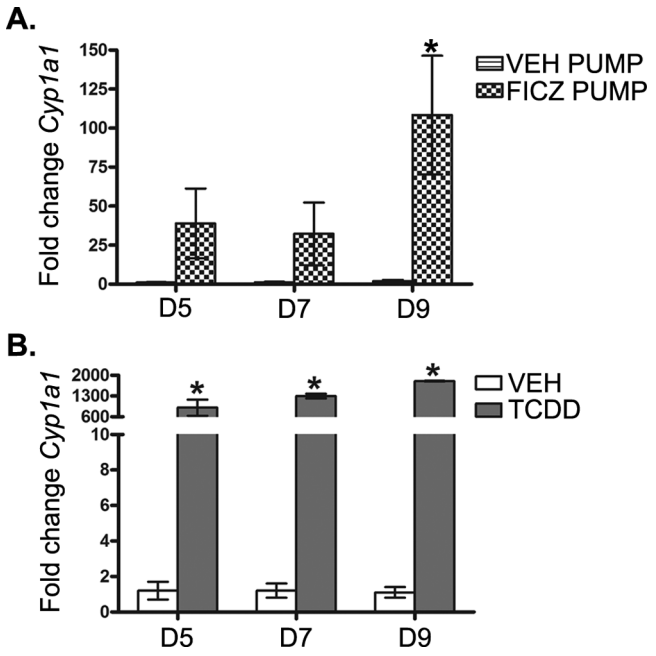

FIG. 4. FICZ-mediated AhR activation is prolonged through micro-osmotic pump implantation. Female C57BL/6 mice were treated with vehicle (VEH) or TCDD as described in Figure 1 or surgically implanted with subcutaneous micro-osmotic pumps loaded with either vehicle control (45% HPβCD) or FICZ released at a concentration of 10 µg/kg body weight/h. Mice were infected as described in Figure 1, 1 day after implantation or gavage. On the indicated days postinfection, RNA was isolated from lung and qRT-PCR was performed on RNA isolated from lungs of (A) mice implanted with FICZ-loaded (FICZ PUMP) and vehicle-loaded (VEH PUMP) micro-osmotic pumps and (B) TCDD and vehicle-treated mice. The mean fold change (± SEM) in *Cyp1a1* was determined using the $2^{-\Delta\Delta CT}$ method with *L13* as the control gene (n = 2–9 mice per treatment group per day). An * indicates a significant difference in fold change *Cyp1a1* expression compared with the vehicle control mice sacrificed on the same day of infection ($p \leq .05$). Abbreviations: AhR, aryl hydrocarbon receptor; FICZ, 6-formylindolo[3,2-b]carbazole; HPβCD, hydroxypropyl-β-cyclodextrin; qRT-PCR, quantitative reverse transcription-PCR; TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin.

infection. Mice implanted with FICZ-loaded pumps had higher expression of *Cyp1a1* compared with mice that were implanted with pumps loaded with vehicle. Moreover, *Cyp1a1* induction was sustained up to 9 days postinfection (Fig. 4A). Thus, the induction of Cyp1a1 was qualitatively similar in duration to that caused by a single oral dose of TCDD (Fig. 4B).

Using this approach, we investigated whether prolonged AhR activation with FICZ, implanted in micro-osmotic pumps, mimics the immunomodulatory effects of TCDD-mediated AhR activation. In contrast to a single dose of TCDD, continuous FICZ dosing did not cause a significant difference in neutrophil frequency (percent or number) in the lungs of infected mice compared with infected mice implanted with vehicle-loaded pumps (Fig. 5A). Similarly, iNOS levels in the lung were unchanged following prolonged AhR activation with FICZ during infection (Fig. 5B). The effect of prolonging AhR activation by FICZ on $CD8^+$ T-cell responses was also evaluated. Both the percentage and number of influenza-specific

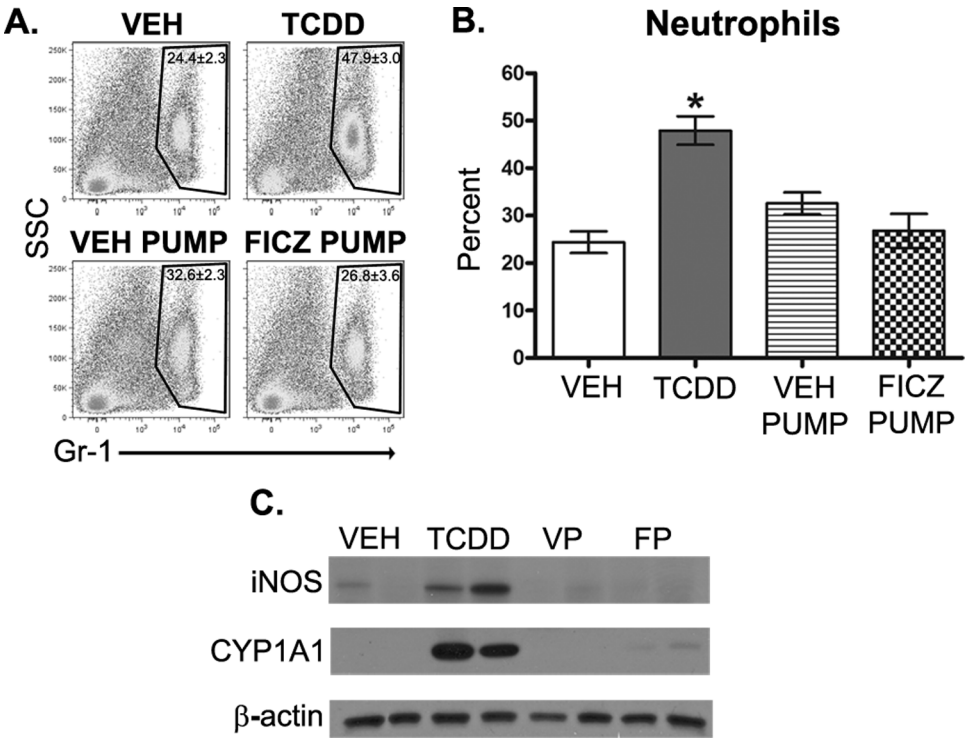

FIG. 5. Prolonged AhR activation with FICZ does not alter neutrophilia or iNOS levels in the infected lung. Mice were treated and infected as described in Figure 4 (n = 4–8 mice per treatment group). A, On day 7 postinfection, immune cells were isolated from collagenase-digested lung, stained with antibodies against Gr-1 and analyzed by flow cytometry. Representative dot plots depict the percentage of Gr-1$^+$ cells in the lung ± SEM (upper right corner). B, The average percentage of neutrophils in the lung was also determined by performing differential cell counts on hematoxylin and eosin–stained cytospins of lung-derived immune cells (n = 4–8 mice per treatment group). C, Lung from mice treated orally with vehicle (VEH), TCDD, implanted with vehicle pump (VP), and FICZ pump (FP) were homogenized, proteins subjected to SDS-PAGE, and probed for iNOS and CYP1A1 by immunoblotting. β-Actin levels were assessed as a control. Blots show 2 representative samples from each treatment group (n = 4–8 mice per treatment group). An * indicates a significant difference compared with vehicle control ($p \leq .05$). Abbreviations: AhR, aryl hydrocarbon receptor; FICZ, 6-formylindolo[3,2-b]carbazole; iNOS, inducible nitric oxide synthase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TCDD, 2,3,7,8 tetrachlorodibenzo-p-dioxin.

CD8$^+$ T cells were reduced by TCDD but were not altered in infected mice implanted with FICZ-loaded pumps (Figs. 6A and 6B). Additionally, the number of CTL and IFNγ$^+$CD8$^+$ T cells remained unchanged following FICZ-pump treatment (Figs. 6C and 6D). Thus, even with continuous FICZ exposure, these metrics of host response to influenza virus infection were unaffected.

Prolonged FICZ Dosing in Influenza Virus–Infected Cyp1a1-Deficient Mice Does Not Enhance Pulmonary Inflammation But Causes Slight Reductions in CD8$^+$ T-Cell Responses

One explanation for these findings is that although the pumps prolong the presence of FICZ *in vivo*, FICZ is still readily metabolized by CYP1A1 (Wei *et al.*, 1998, 2000). If so, then a comparison of immune modulation when AhR is activated *in vivo* by these 2 distinct ligands may be skewed by the rapid metabolism of FICZ versus the poor metabolism of TCDD. In order to evaluate the influence of FICZ metabolism, we used *Cyp1a1*-deficient mice (*Cyp1a1*$^{-/-}$ mice). Specifically, *Cyp1a1*$^{-/-}$ mice were implanted with either FICZ or vehicle-loaded pumps 1 day before infection. When pulmonary inflammation was evaluated at day 7 postinfection, no difference in neutrophil frequency or iNOS levels were observed in the lungs of *Cyp1a1*$^{-/-}$ mice that were implanted with FICZ pumps compared with vehicle controls (Figs. 7A and 7B). In contrast, *Cyp1a1*$^{-/-}$ mice treated with TCDD had significantly enhanced pulmonary neutrophilia and elevated iNOS levels, similar to TCDD-treated wild-type mice (data not shown). Additionally, mice treated with FICZ-loaded pumps exhibited infection-associated morbidity and mortality that were more similar to vehicle-treated controls, whereas enhanced mortality was observed in the TCDD-treated group (Fig. 7C).

CD8$^+$ T-cell responses in infected *Cyp1a1*$^{-/-}$ mice were also examined. In the absence of CYP1A1-mediated metabolism, prolonged FICZ treatment slightly diminished the magnitude of the CD8$^+$ T-cell response to infection. Specifically, the percentage of IFNγ$^+$CD8$^+$ T cells were reduced in FICZ-treated *Cyp1a1*$^{-/-}$ mice compared with vehicle controls, although this did not reach statistical significance (p = .07, Fig. 7D). Slight reductions in the frequency of NP-specific CD8$^+$ T cells were also observed in FICZ-treated mice (p = .06, Fig. 7E).

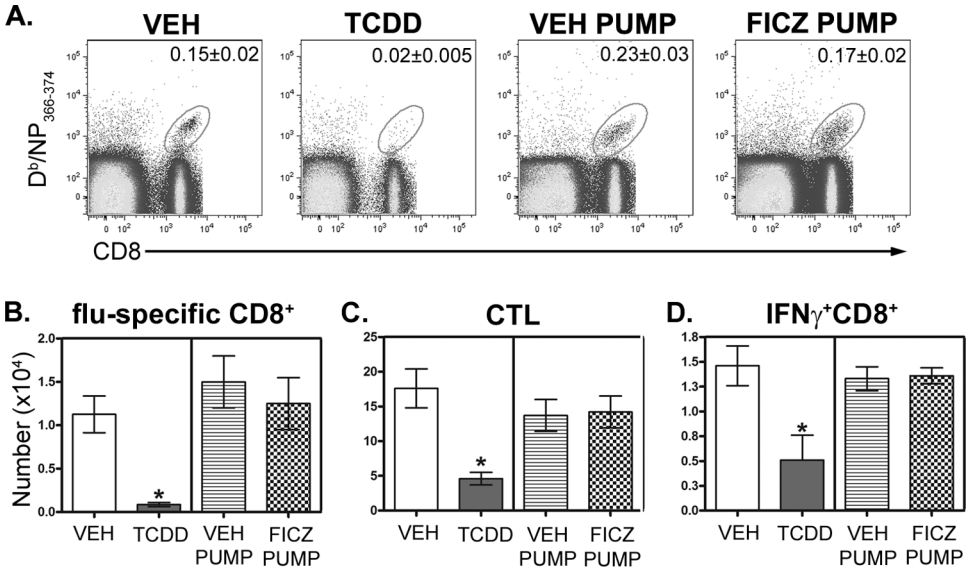

FIG. 6. Prolonged AhR activation with FICZ does not suppress the CD8⁺ T-cell response to influenza virus infection. Mice were treated and infected as described in Figure 4. On day 9 postinfection, MLN cells were collected and stained with MHC class I tetramers and antibodies as described in the Materials and Methods section. A, Representative dot plots depict the frequency of $D^b/NP_{366–374}$-specific (virus-specific) CD8⁺ T cells obtained from mice treated orally with vehicle (VEH), TCDD, implanted with vehicle pump (VEH PUMP), and FICZ-loaded pump (FICZ PUMP). The number on each plot indicates the percentage of total MLN cells (± SEM). Bar graphs show the average number of (B) virus NP-specific CD8⁺ T cells, (C) $CD44^{hi}CD62L^{lo}CD8^+$ (CTL), and (D) IFNγ⁺CD8⁺ T cells in each treatment group (± SEM) (n = 8–10 mice per treatment group). An * indicates a significant difference compared with vehicle control ($p \leq .05$). Abbreviations: AhR, aryl hydrocarbon receptor; FICZ, 6-formylindolo[3,2-b]carbazole; IFN, interferon; MHC, major histocompatibility complex; MLN, mediastinal lymph node; NP, nucleoprotein; TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin.

Consistent with these observations in the lymph nodes, infected FICZ-treated *Cyp1a1*$^{-/-}$ mice had significantly fewer lymphocytes in their lungs compared with vehicle-treated *Cyp1a1*$^{-/-}$ mice (Fig. 7F). However, the diminution of these responses in *Cyp1a1*$^{-/-}$ mice was not as substantial as the change caused by a single dose of TCDD 1 day prior to infection (data not shown). These findings suggest that although innate immune responses may be unaffected by reduced metabolism of and prolonged exposure to FICZ, decreasing FICZ metabolism may attenuate the CD8⁺ T-cell response to influenza virus infection.

DISCUSSION

The AhR is a fascinating molecule, as it is a ligand-activated transcription factor triggered by a broad spectrum of structurally diverse ligands (Nguyen and Bradfield, 2008). Evidence from numerous research groups demonstrates that it plays a role in the development and function of the immune system. However, it is not completely understood how different AhR ligands modulate immune function. In the work presented here, we compared the consequences of AhR activation by TCDD and FICZ on the 3 distinct components of the response to infection with a common respiratory virus. Rather than skew responses to infection in a manner that is opposite of TCDD, FICZ had no effect whatsoever. Specifically, whether FICZ was administered orally in a single bolus, given twice daily, or released continuously over the course of infection, pulmonary neutrophilia, iNOS levels in the infected lung, and the clonal expansion and differentiation of CD8⁺ T cells were not altered. One interpretation of these findings is that AhR-dependent pathways in certain immune cell populations, such as CD4⁺ T cells, could be more sensitive to differential AhR ligation than others. In the EAE model, CD4⁺ T cells are one of the critically important cell types involved in progression of the disease (Chen and Shannon, 2013). However, CD4⁺ T cells are *not* required for host resistance to most primary influenza virus infections (Belz *et al.*, 2001). Instead, viral clearance and host resistance are heavily dependent on the response of CD8⁺ T cells and neutrophils (Tate *et al.*, 2009; Topham *et al.*, 1997). Moreover, although an AhR-mediated reduction in the CD8⁺ T-cell response to infection requires AhR in hematopoietic cells, increased neutrophilia and iNOS levels in the infected lung are due to AhR activation in nonhematopoietic cell lineages (Lawrence *et al.*, 2006; Neff-LaFord *et al.*, 2007; Teske *et al.*, 2008; Wheeler *et al.*, 2013). Therefore, it is possible that differential AhR activation could have varying effects dependent upon the specific cell type in which the receptor is activated. Thus, although the FICZ-mediated immunomodulation, such as that observed in the EAE model, may be dependent on AhR activation within CD4⁺ T cells, the immune perturbation caused by AhR activation during influenza virus infection may be dependent on activation of the AhR in non-CD4⁺ cell types.

Other discoveries support the idea that different AhR ligands may have varying effects depending on the model system.

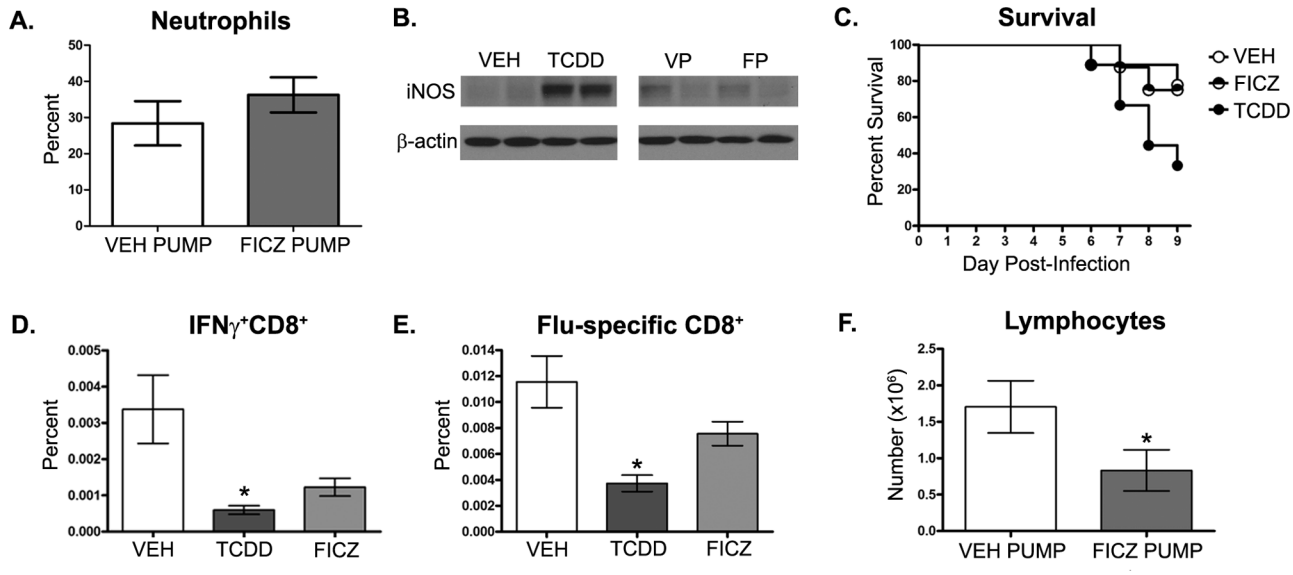

FIG. 7. *Cyp1a1*$^{-/-}$ mice have slight reductions in CD8$^+$ T-cell responses to influenza virus infection, but not in lung neutrophil or iNOS levels, following prolonged AhR activation with FICZ. Cyp1a1$^{-/-}$ mice were treated and infected as described in Figure 4. A, On day 7 postinfection, mice implanted with either vehicle (VEH PUMP) or FICZ-loaded pumps (FICZ PUMP) were sacrificed and immune cells were isolated from collagenase-digested lung and the average percentage of neutrophils were determined by performing differential cell counts on hematoxylin and eosin–stained cytospins of lung-derived immune cells (n = 4–8 mice per treatment group). B, On day 7 postinfection, lungs from mice implanted with vehicle pump (VP) or FICZ pump (FP) were homogenized, proteins subjected to SDS-PAGE, and probed for iNOS. β-Actin levels were assessed as a control. Representative samples from orally treated VEH and TCDD mice were used at negative and positive controls, respectively. Blots show 2 representative samples from each treatment group (n = 4–8 mice per treatment group). C, Survival was monitored up to 9 days postinfection in orally treated VEH, TCDD, or mice implanted with pumps loaded with FICZ (FICZ PUMP) (D and E). MLN were isolated from mice on day 9 postinfection and stained with antibodies for flow cytometry as described in Figure 2. Bar graphs show the average percentage (± SEM) of (D) IFNγ$^+$CD8$^+$ T cells and (E) influenza virus NP-specific CD8$^+$ T cells in each treatment group (n = 8–10 mice per treatment group). F, On day 7 postinfection, immune cells were isolated from collagenase-digested lung and the average percentage of lymphocytes were determined by performing differential cell counts on hematoxylin and eosin–stained cytospins of lung-derived immune cells (n = 4–8 mice per treatment group). An * indicates a significant difference compared with vehicle control ($p \leq .05$). Abbreviations: AhR, aryl hydrocarbon receptor; FICZ, 6-formylindolo[3,2-b]carbazole; IFN, interferon; iNOS, inducible nitric oxide synthase; MLN, mediastinal lymph node; NP, nucleoprotein; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin.

For example, during occular herpes simplex virus (HSV) infection, inflammatory lesions and total CD4$^+$ T-cell number were reduced in TCDD-treated mice compared with infected controls; yet, TCDD skewed differentiation of CD4$^+$ T cells toward T_{regs} (Veiga-Parga *et al.*, 2011). However, treatment with FICZ failed to cause any change in the immune response to HSV. In another study, FICZ had no effect on peanut allergen sensitization, whereas TCDD significantly modulated multiple parameters of the allergic response, including antibody and cytokine production, and the frequency of CD4$^+$CD25$^+$Foxp3$^+$ cells (Schulz *et al.*, 2012). In contrast to these reports of null effects, FICZ reduced pulmonary eosinophilia and Th2 cytokines in the lungs of mice in an ovalbumin (OVA) model of allergic asthma (Jeong *et al.*, 2012) in a manner quite similar to reported consequences of TCDD-mediated AhR activation in OVA and house dust mite allergy models (Luebke *et al.*, 2001; Nohara *et al.*, 2002). Collectively, these findings suggest that immune responses are sensitive to differential AhR activation, but differences in the consequences may be dependent on not only key cell types involved but also site of antigen challenge and type of infection or insult.

Another potential explanation for dissimilarities in modulation of the response to influenza virus by FICZ versus TCDD is the duration of AhR activation. We addressed this possibility by simultaneously prolonging FICZ's presence using microosmotic pumps and reducing the metabolism of FICZ using *Cyp1a1*$^{-/-}$ mice. In the absence of CYP1A1, TCDD still altered immune responses to influenza virus, suggesting that the induction of CYP enzymes and changes in immune function occur by two independent, AhR-mediated pathways. This is consistent with earlier reports, in which thymic atrophy and dysregulated leukocyte distribution were observed, but hepatotoxicity was attenuated, in *Cyp1a1*$^{-/-}$ mice given a very high dose of TCDD (Uno *et al.*, 2004). Yet, our data suggest that duration of activation alone does not account for immunomodulatory differences between these two AhR ligands, given that FICZ still had no effect on neutrophil frequency or lung iNOS levels in *Cyp1a1*$^{-/-}$ mice. However, there was a modest reduction in the response of CD8$^+$ T cells in *Cyp1a1*$^{-/-}$ mice treated with FICZ, suggesting that TCDD's longer lasting effects on AhR activation can be mimicked by continual FICZ treatment if its metabolism is reduced. This idea is consistent with prior reports that have shown differential sensitivities among immune cell subsets to AhR activation. For example, during infection with influenza virus, enhanced pulmonary neutrophilia waned faster than the suppressed lymphocyte responses with decreasing

doses of TCDD (Vorderstrasse *et al.*, 2003). In other words, suppression of lymphocyte responses may be slightly more sensitive to AhR modulation than enhanced lung inflammation. Thus, when clearance of FICZ was reduced in *Cyp1a1*$^{-/-}$ mice, the effects on T cells were evident even though, at the dose of FICZ administered, pulmonary inflammation was not altered.

Another observation reported herein is that, although the pattern of increased *Cyp1a1* induction by FICZ when implanted in pumps was qualitatively similar in duration to that induced by a single oral dose of TCDD, the overall level of *Cyp1a1* expression was approximately 10-fold lower. FICZ and TCDD have high affinities for the AhR (FICZ K_d = 0.07nM, TCDD K_d = 0.48nM) (Rannug *et al.*, 1987); thus, binding affinity *per se* is unlikely to explain this difference. Our data are similar to findings from Shulz *et al.* (2012), in which TCDD induced *Cyp1a1*, *1a2*, *1b1* mRNA, and EROD activity at levels much greater than FICZ. There are several reasons why FICZ, while having a slightly higher affinity for AhR than TCDD, may not induce AhR target genes at levels similar to TCDD. It has been posited that the intrinsic properties of structurally distinct ligands influence downstream consequences of AhR activation (Denison *et al.*, 2011). This phenomenon has been reported for other ligand-activated nuclear receptors, in which distinct ligands result in differences in receptor conformation, which are thought to influence the "quality" of activation through recruitment of various coactivators and transcription proteins (Jin and Li, 2010). Therefore, it is plausible that activation of the AhR with structurally distinct ligands induces different conformational states. Although a crystal structure has not yet been elucidated for the AhR, this idea is supported by some studies using homology modeling (Pandini *et al.*, 2007). Also, variation in coactivator recruitment and histone modifications at AhR target genes have been reported for different AhR ligands (Hestermann and Brown, 2003; Ovesen *et al.*, 2011; Pansoy *et al.*, 2010). It has also been shown that compounds with different toxic equivalency factors have strikingly similar histone marking profiles on genes downstream of AhR activation (Ovesen *et al.*, 2011). Additionally, mouse fibroblasts treated with another AhR ligand, 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), had qualitatively similar patterns of gene induction compared with TCDD (Henry *et al.*, 2010). However, although the same genes were induced by ITE and TCDD in this study, differences in the kinetics of the response were observed. Related to this concept is the fact that different ligands are metabolized at different rates. Thus, disparities in gene expression and downstream functional consequences of AhR activation by FICZ versus TCDD could be due to the rapid metabolism of FICZ. Although *in vitro* studies reveal that CYP1A1 is the primary enzyme responsible for FICZ metabolism, CYP1B1, CYP1A2, and possibly other enzymes also affect its metabolism (Mukai and Tischkau, 2007). Indeed, it is important to realize that complete *in vivo* pharmacokinetic data on FICZ remain to be fully developed. Hence, complete inhibition of FICZ metabolism *in vivo* may be difficult to achieve without an improved understanding of the *in vivo* metabolism of this tryptophan photodegradation product. Our findings add to the body of knowledge about *in vivo* metabolism and biological effects of FICZ. When combined with reports from others (Wei *et al.*, 1998; Wincent et al., 2009, 2012), this information collectively demonstrates that differences in the nature of AhR activation and ligand metabolism could affect the manner in which different ligands modulate pathophysiology *in vivo.*

As we consider the idea of targeting AhR signaling in connection with treating human disease, these studies emphasize that one must be cautious about assuming that an AhR ligand's actions at the cellular level will necessarily translate to that compound's ability to modulate complex pathophysiological processes *in vivo*. Moreover, even when there is compelling evidence that a particular AhR ligand can alter disease course *in vivo*, it is critically important to realize that the immunomodulatory efficacy of this ligand may depend upon, and indeed vary among, specific *in vivo* contexts. A better understanding of how different AhR ligands alter immune function in multiple model systems will shed light on not only mechanisms through which the AhR could be manipulated as a therapy for disease but will also inform public health risks of environmental ligands and help to reveal the endogenous functions of this enigmatic receptor.

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